academicJournals

Vol. 16(16), pp. 895-907, 19 April, 2017
DOI: 10.5897/AJB2016.15858
Article Number: BF45A9463779
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Genetic diversity in Nepalese population of *Swertia chirayita* (Roxb. Ex Fleming) H. Karst based on inter-simple sequence repeats (ISSR) markers

Surendra Neupane[1,2]*, Jaishree Sijapati[1], Tribikram Bhattarai[2] and Sangita Shrestha[1]

[1]Molecular Biotechnology Unit, Nepal Academy of Science and Technology (NAST), Khumaltar, Lalitpur, Nepal.
[2]Central Department of Biotechnology, Tribhuvan University, Kirtipur, Kathmandu, Nepal.

Received 28 December, 2016; Accepted 20 March, 2017

Swertia chirayita **is a highly valued but vulnerable medicinal plant species of Nepal. Its populations are declining in natural habitats due to over exploitation. Twenty-seven inter-simple sequence repeats (ISSR) primers were used to assess the genetic diversity and population genetic structure of 42 genotypes representing six natural populations of *S. chirayita*, from Nepal. Of the total 479 bands amplified by 27 ISSR primers, 473 (98.18%) were polymorphic, indicating very high level of genetic diversity at species level. Percentage polymorphism value for different primers ranged from 83.3 to 100% with an average of 98.18%. Polymorphism information content (PIC) value ranged from 0.88 to 0.93 with an average of 0.91. Cluster analysis performed with NTSYS pc statistical package using Jaccard's similarity coefficients generated from ISSR binary data matrix showed that, all 27 ISSR primers separated 42 individuals into two major clusters and six sub clusters at the similarity level of 0.24. The average value of Nei's genetic diversity (H) and Shannon's information index (I) equaled 0.276 and 0.423, respectively at species level. The coefficient of genetic differentiation (G_{ST}) amongst populations of *S. chirayita* was found to be high (0.548) with restricted gene flow (N_m=0.4829). Analysis of molecular variance showed that genetic diversity within populations is slightly higher (50.9%) than among populations (47.6%). The present genetic diversity assessment of *S. chirayita* populations has been of immense importance to understand the cause of its vulnerability and has furnished valuable insights for its conservation and sustainable utilization.**

Key words: Polymerase chain reaction, inter-simple sequence repeats (ISSR), genetic diversity, polymorphism, population genetics.

INTRODUCTION

Nepal is home to medicinal and aromatic plants (MAPs) diversity and over 1950 medicinal plants have been stated till date (Ghimire, 2008). Among them, 30 species of *Swertia* (Gentianaceae), including varieties are

*Corresponding author. E-mail: suren.neupane04@hotmail.com. Tel: +1(605)-690-7678.

reported in Nepal (Press et al., 2000). *Swertia chirayita* (Roxb. ex Flem) Karst, the most valuable and dominant species in trade, is indigenous to the temperate Himalayas and is abundant in 54 districts in Nepal (Barakoti et al., 2013) throughout the altitude ranging from 600 to 5600 m (Rijal, 2010). Because of its high need in national and international markets, natural populations have been threatened and species as such has been considered vulnerable medicinal plant in Himalayan region including Nepal (Nepal, 2004; Purohit et al., 2013). Together with *S. chirayita*, eight other species of *Swertia* [*Swertia angustifolia* Buch.-Ham ex D. Don, *Swertia dilatata* C. B. Clarke, *Swertia nervosa* (G. Don) C. B. Clarke, *Swertia racemosa* (Griseb.) C.B. Clarke, *Swertia ciliata* (D. Don ex G. Don) B. L. Burtt, *Swertia multicaulis* D. Don, *Swertia alata* (Royle ex D. Don) C.B. Clarke and *Swertia tetragona* Edgew] are also traded for their medicinal properties with the name 'Chiraito' or 'Chiretta' (Barakoti, 2002). Among these nine species, *S. chirayita* is considered superior in medicinal quality and is in high demand in trade nationally and internationally (Barakoti, 2002; Joshi, 2008). Shrestha et al. (2016) have reported that *S. chirayita* is the mostly used species by the indigenous people of Sankhuwasabha district of Nepal proving the efficacy and importance in ethnomedical research. Nepal trades more than 45% of the world's total traded volume of *S. chirayita* (Barakoti, 2004). Nevertheless, Nepal uses only 1% of this volume and the rest is exported to various countries including India, Italy, France, Switzerland, Sri Lanka, Bangladesh, Pakistan, China, Germany, Singapore and the United States of America (Phoboo et al., 2008). Numerous studies have reported many chemical compounds in *S. chirayita* such as chiratinin, terpenoids, iridoids gentianine, amarogentin, amaroswerin, xanthones, secoiridoid, glycosides and urosilic acid (Bajpai et al., 1991; Joshi and Dhawan, 2005; Khanal et al., 2015; Kumar and Chandra, 2015; Kshirsagar et al., 2016, 2017). These compounds and their derivatives possess antihepatotoxic, antileishmanial, anticarcinogenic, antioxidant, anti-inflammatory, antidiabetic, antimalarial and antihelmithic properties (Bajpai et al., 1991; Ray et al., 1996; Saha et al., 2004; Iqbal et al., 2006; Balaraju et al., 2009; Chen et al., 2011; Nagalekshmi et al., 2011; Phoboo et al., 2013; Zhou et al., 2015; Lad and Bhatnagar, 2016). Recently, Tupe et al. (2017) have reported that *S. chirayita* showed antiglycating activity which plays crucial role in antidiabetic effects. Also, *S. chirayita* possess less inhibitory effect on the drug metabolizing isoenzymes CYP3A4 and CYP3D6 as well as inhibits the β-glucuronidase and helps in hepatoprotection (Ahmmed et al., 2016; Karak et al., 2017).

The knowledge of plant genetic diversity in their natural habitat, sustainable utilization, is important for efficient management of plant genetic resources (Mondini et al., 2009). Thus, information on genetic diversity and geographical distribution of plant species in wild conditions are essential for formulating conservation strategy (Wang et al., 2011). Analysis of the genetic diversity and population genetic structure using various molecular marker techniques is necessary to endorse the 'vulnerable' status of any species (Godt and Hamrick, 1998). Thus, molecular tools play important role in exploring genetic diversity in endangered species for the formulation conservation strategies (Kim et al., 2005).

PCR-based inter-simple sequence repeats (ISSR) markers have been widely employed for studying population genetics of various plant species, including several medicinal plants such as *Solanum tuberosum*, *Neopicrorhiza scrophulariiflora* and *Dendrobium* spp. (Bornet et al., 2002; Wang et al., 2009; Liu et al., 2011). Limited molecular studies have been carried out in *Swertia* species of the world. In a study including ISSR markers, 98.7% polymorphism was found among 19 genotypes of *Swertia* spp. (13 of *S. chirayita* and 2 each of *Swertia cordata*, *Swertia paniculata* and *Swertia purpurascenes*) collected from the temperate Himalayas of India (Joshi and Dhawan, 2007). In the investigation of endangered endemic species, *Swertia przewalskii* of the Qinghai-Tibet plateau using RAPD and ISSR analysis, Zhang et al. (2007) observed the significant genetic differentiation based on different measures including analysis of molecular variance (AMOVA) (52% for RAPD and 56% for ISSR). Misra et al. (2010) used amplified fragment length polymorphism (AFLP) to produce DNA fingerprints for *Swertia* spp. In the study, 19 accessions (two of *S. chirayita*, three of *Swertia augustifolia*, two of *Swertia bimaculata*, five of *S. ciliata*, five of *S. cordata* and two of *S. alata*) from India were used in the study by employing 46 selected AFLP primer pairs. The species-specific markers were identified for all six *Swertia* spp. which can be used to authenticate drugs. Another study revealed polymorphism of up to 99% among various species of *Swertia* using 16 ISSR primers (Tamhankar et al., 2009). They found *Swertia lurida* to be the closest to *S. chirayita*. Another study on *Swertia tetraptera* showed high genetic diversity within species and low genetic diversity among populations (Yang et al., 2011). Also, Samaddar et al. (2015) reported on some RAPD markers that can be used for fingerprinting analysis and implementation of genetic diversity study on *Swertia* spp.

In the context of Nepal, initiation of molecular characterization of *Swertia* spp. has been reported with the phylogeny of 11 Nepalese *Swertia* spp. such as *S. augustifolia, S. chirayita, S. ciliata, S. dilatata, S. lurida, Swertia macrosperma, S. multicaulis, S. nervosa, S. paniculata, Swertia pedicellata* and *S. racemosa* (Joshi, 2008, 2011). The internal transcribed spacer (ITS) and chloroplast (*trnL-F*) regions were analyzed together with distance, parsimony and Bayesian analysis. The result indicated that ITS fragment can be used in identification as a barcoding marker for *Swertia*. The optimization of the RAPD-PCR conditions (Shrestha et al., 2011) and

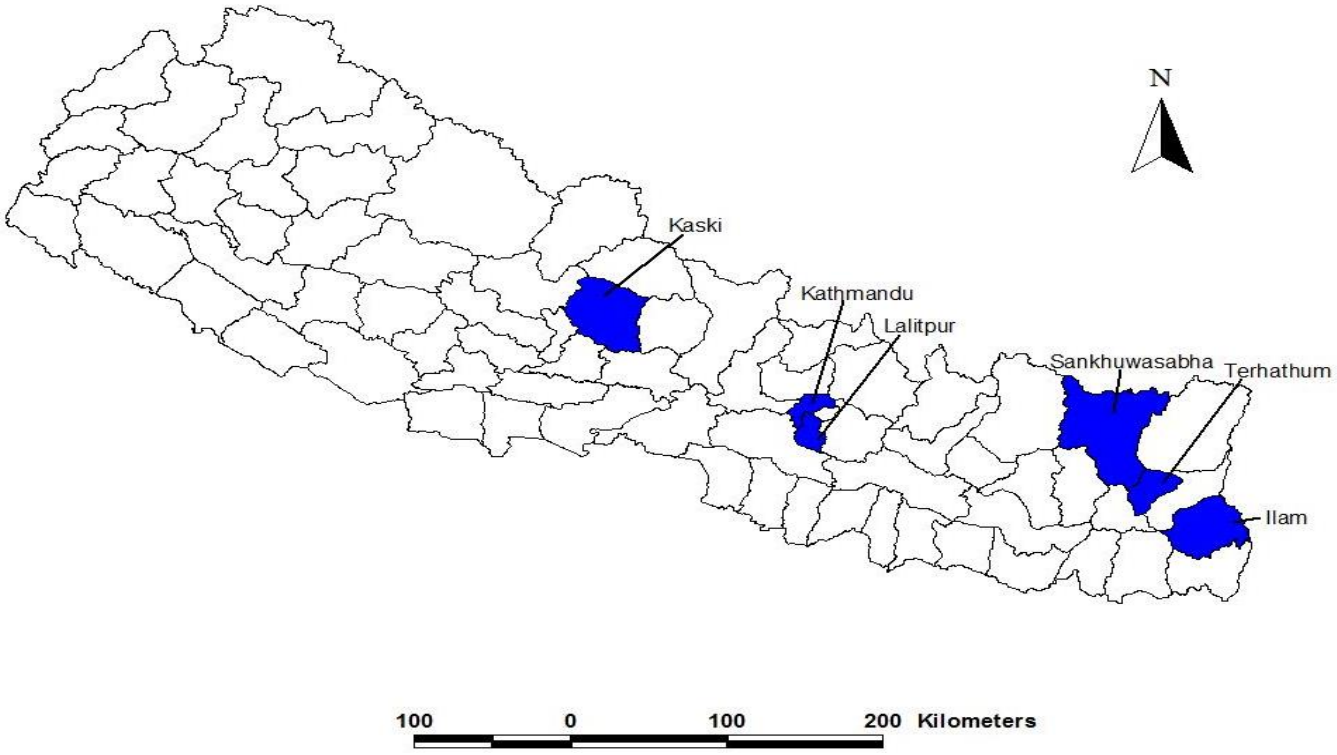

Figure 1. Geographic locations of collected *Swertia chirayita* of Nepalese populations under study.

genetic diversity of 34 accessions of *S. chirayita* including six outlier species were assessed in Nepal (Shrestha et al., 2013). Of the total 285 amplified bands scored for *S. chirayita*, 263 (92.8%) were polymorphic.

The objectives of this study were to assess the genetic diversity, unravel the genetic variation within and among populations and provide future recommendations necessary for conservation and sustainable utilization of *S. chirayita*, the highly valuable and vulnerable medicinal plant species of Nepal.

MATERIALS AND METHODS

Genetic material and genotypic data

Forty-two samples of *S. chirayita* were collected in silica gel from six different locations of Nepal: nine from Phulchowki, four from Kaski, six from Sankhuwasabha, eight from Terhathum, seven from Nagarjun, eight from Ilam along with four outlier accessions from Kaski and Sankhuwasabha districts (Figure 1, Plate 1 and Table 1). Genomic DNA was extracted by using hexadecyltrimethyl ammonium bromide (CTAB) method (Graham et al., 1994). Quantification and purity assessments of DNA were assessed by using a UV Biophotometer (EPPENDORF AG 22331, Germany). ISSR-PCR reaction parameters were optimized in 20 µl reaction volume containing 50 ng of genomic DNA, 2.4 µl of $MgCl_2$ (3.0 mM), 2.0 µl of 10X Taq polymerase reaction buffer [100 mM Tris-HCl (pH 8.8 at 25°C), 500 mM KCl, 0.8% (v/v) Nonidet P40], 1 U Taq DNA polymerase (Thermo Scientific company), 0.3 mM of dNTPs and 0.6 µM of each primer.

PCR cycling conditions described by Tamhankar et al. (2009) produced finest ISSR profiles for *S. chirayita*. The PCR program comprised of an initial denaturation step at 94°C for 2 min followed by 45 cycles of 95°C for 30 s, 51°C for 45 s and 72°C for 2 min and final extension of 72°C for 5 min. Amplification of DNA was performed using BIOER Xp thermal cycler [BIOER Technology Co. LTD, Taiwan, China]. The PCR amplified ISSR fragments were assessed by gel electrophoresis using 1.5% agarose (Promega Co.) in 1X TAE stained with ethidium bromide (10 mg/ml solution, Promega Co.) buffer at 50 V (4.2 V/cm) in EMBI TEC (Santiago, CA) gel tank for 2 h 30 min. The gel documentation was done using Gel Doc system (IN GENIUS, Syngene Bioimaging, UK). 100 ISSR primers (UBC primer, University of British Columbia, Oligonucleotide Synthesis Laboratory, Vancouver, British Columbia, Canada) were employed to screen against DNA of *S. chirayita* from Nagarjun. Twenty-seven UBC primers (Table 2) that engendered reproducible and scorable bands were selected for the ISSR profiling. Both polymorphic and monomorphic bands were scored as “1” for presence and “0” for absence whereas, the failure in amplification was scored as “9”, as an indicator of missing data (Jaccard, 1908). The size of the ISSR-PCR products was determined using Gene rulerTM100 bp plus DNA ladder (Thermo Scientific Company) (Plate 1).

Genetic diversity and clustering analysis

The reproducible bands scored across all samples were included in the analysis. The binary data matrix was investigated using MS-Excel 2007 for the assessment of total number of bands (TNB), number of polymorphic bands (NPB), percent polymorphism (PP), polymorphic information content (PIC), band informativeness (I_B), and resolving power (R_P) for each primer. These are calculated by,

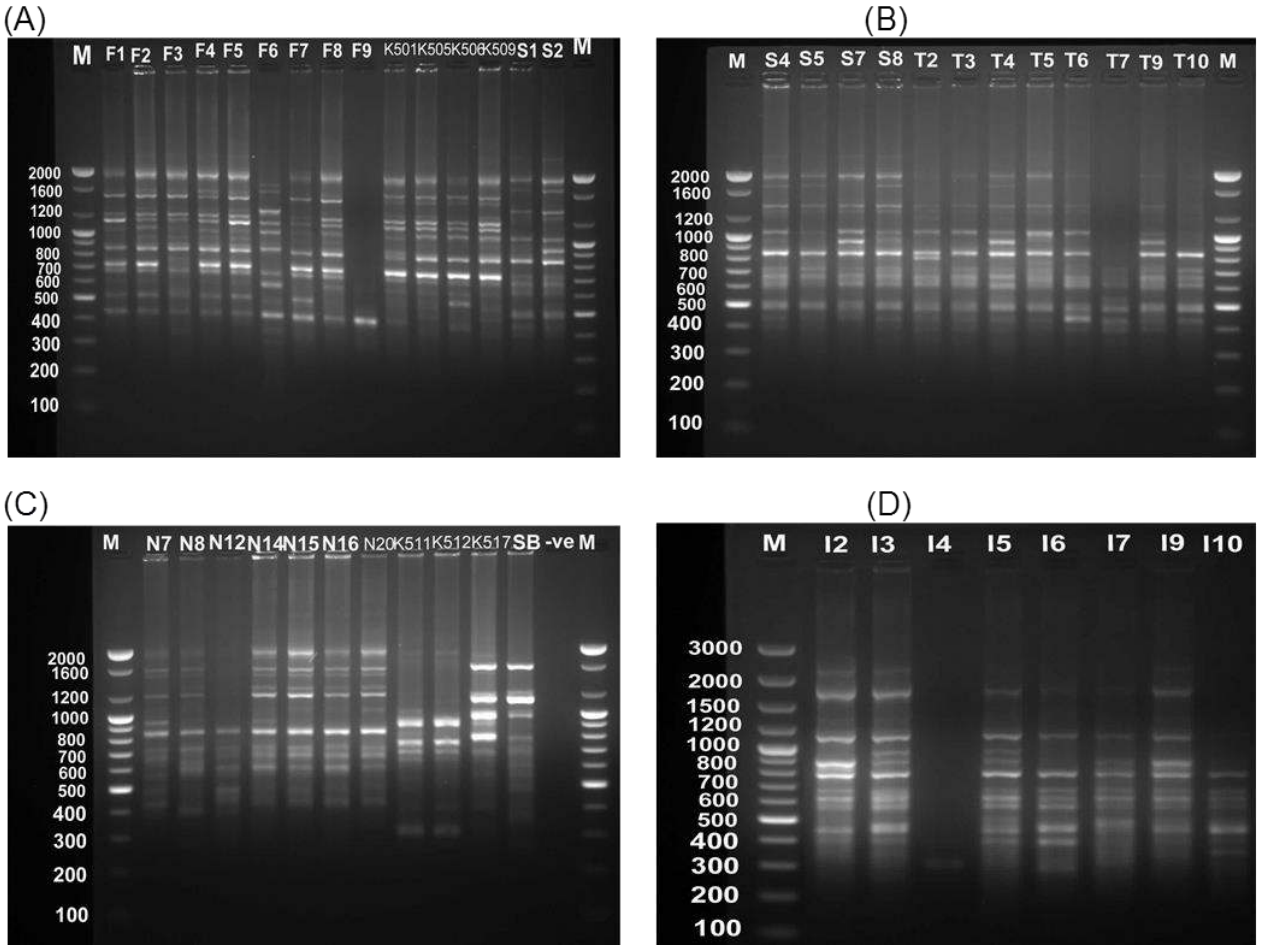

Plate 1. ISSR profile generated by primer UBC 842 for *Swertia chirayita* populations. Lanes marked with M are 100plus DNA ladder. (A) Lanes F1-F9 from Phulchowki, K501-K509 from Kaski and S1-S2 from Sankhuwasabha; (B) S4-S8 from Sankhuwasabha and T2-T10 from Terhathum; (C) N7-N20 from Nagrajun and K511-K517, SB as outliers (*Swertia sp.*); (D) I2-I10 from Ilam.

PP = NPB/TNB engendered by each primer; PIC = 1- $\Sigma(P_{ij})^2$, where P_{ij} denotes the frequency of the ith pattern revealed by the jth primer added throughout patterns revealed by the primers (Botstein et al., 1980); Rp = ΣI_B, where I_B is the band informativeness with IB = 1-[2×(0.5-P)], where P denotes the proportion of accessions containing the band (Prevost and Wilkinson, 1999). The similarity indices were computed using a similarity for qualitative data (SIMQUAL) computer algorithm via NTSYS-PC (Numerical Taxonomy and Multivariate System, version 2.21i, Exeter software; Setauket, New York, USA).

The program POPGENE 1.31 was used to estimate intra and inter-population genetic variation. The parameters used were Nei's gene diversity index (H), Shannon's information index (I), the observed number of alleles (Na) and the effective number of alleles (Ne). Nei's gene diversity statistics along with total genetic diversity (H_T), genetic diversity within populations (H_S), and the extent of genetic differentiation among populations (Gst) was estimated to study the genetic construction (Nei, 1978). Gst was calculated using formula as (H_T - H_S)/H_T. The gene flow among populations (Nm) was calculated using formula (1 - G_{st})/4G_{st} by Slatkin and Barton (1989). GenAlEx ver 6.5 was used for analysis of molecular variance (AMOVA) and Mantel test done to estimate the genetic variation among and within populations (Peakall and Smouse, 2012). AMOVA was tested by nonparametric randomization tests using 999 permutations in variation attribute. 3D plot of the distribution of all *S. chirayita* accessions was constructed with the analysis of Eigen vector for the illustration of variation as compared to the dendrogram using Jaccard's similarity matrix using NTSYS-PC.

Principal coordinates analysis (PCA) was performed through Nei's genetic distance (Nei, 1978) matrix of populations using GenAlEx software (Peakall and Smouse, 2012). Finally, F_{st} was calculated employing Tools for Population Genetic Analysis (TFPGA) through 1000 permutations (Miller, 1997).

RESULTS

ISSR analysis

The total number of bands (TNB), number of polymorphic bands (NPB), percentage polymorphism (PP), amplicon size range, PIC, I_B and R_P values of the 27 ISSR primers used to generate ISSR profiles of *S. chirayita* accessions are presented in Table 2. The 27 selected primers

Table 1. Sample details of *S. chirayita* and other outlier species used in the present study (locality, number of samples, altitude, plant accession codes, latitude/longitude of the plant samples collected; C= Central, W= Western, E= Eastern).

District/Locality	Sample size	Altitude (m)	Plant accession codes	Latitude/Longitude
C. Nepal, Lalitpur, Phulchowki, Godavari	9	2100-2150	F1, F2, F3, F4, F5, F6, F7, F8, F9	27.34°N, 85.23°E
W. Nepal, Kaski, Sikles	4	2000-2500	K501, K505, K506, K509	28.21°N, 84.60°E
W. Nepal, Kaski, Sikles	3	2000-2500	K511, K512, K517	28.20°N, 84.59°E
C. Nepal, Kathmandu, Nagarjun, Jamacho,	7	2050-2100	N7, N8, N12, N14, N15, N16, N20	-
E. Nepal, Sankhuwasabha, Lamapokhari-Manlabre	6	2600	S1, S2, S4, S5, S7, S8	27.37°N, 87.78°E
E. Nepal, Sankhuwasabha, Lamapokhari-Manlabre	1	2600	SB	27.37°N, 87.80°E
E. Nepal, Terhathum, Tirkhimti-Gupha Pokhari	8	1500-2800	T2, T3, T4, T5, T6, T7, T9, T10	27.11°N, 87.30°E
E. Nepal, Ilam, Maipokhari	8	-	I2, I3, I4, I5, I6, I7, I9, I10	26.90°N, 87.92°E

Total: 46 samples. Outliers (K511 and K512: *Swertia dilatata* C. B. Clarke and K517 and SB: *Swertia pedicellata* Banerji).

Table 2. Primer sequences, total number of bands (TNB), number of polymorphic bands (NPB), percentage polymorphism (PP), amplicon size range, PIC, I_B and R_P values of the 27 ISSR primers used to generate ISSR profiles of *S. chirayita* populations.

Primers	Primer sequence	TNB	NPB	PP (%)	Amplicon size (bp)	PIC	I_B	R_P
UBC811	GAG AGA GAG AGA GAG AC	17	16	94.12	350-2000	0.914	0.45	8.62
UBC842	GAG AGA GAG AGA GAG AYG	22	22	100	300-2200	0.931	0.58	12.86
UBC 810	GAG AGA GAG AGA GAG AT	19	19	100	250-2000	0.911	0.57	12.00
UBC 820	GTG TGT GTG TGT GTG TC	11	11	100	600-2000	0.859	0.46	5.52
UBC 830	TGT GTG TGT GTG TGT GG	8	8	100	500-2000	0.805	0.43	3.43
UBC812	GAG AGA GAG AGA GAG AA	12	10	83.33	350-2000	0.825	0.31	5.62
UBC 817	CAC ACA CAC ACA CAC AA	15	15	100	400-2100	0.906	0.59	9.43
UBC 809	AGA GAG AGA GAG AGA GG	20	18	90	250-2000	0.927	0.51	11.67
UBC 807	AGAGAGAG AGA GAG AGA GT	16	16	100	400-2100	0.912	0.63	11.3
UBC836	AGA GAG AGA GAG AGA GYA	24	24	100	250-2200	0.938	0.54	13.62
UBC 848	CAC ACA CAC ACA CAC ARG	21	21	100	300-2200	0.916	0.43	9.8
UBC 850	GTG TGT GTG TGT GTG TYC	22	22	100	350-2100	0.937	0.49	11.43
UBC 818	CAC ACA CAC ACA CAC AG	18	18	100	300-2200	0.886	0.36	6.48
UBC 840	GAG AGA GAG AGA GAG AYT	16	16	100	500-3000	0.914	0.51	9.62
UBC 841	GAG AGA GAG AGA GAG AYC	22	22	100	300-2800	0.93	0.58	14.95
UBC 857	ACA CAC ACA CAC ACA CYG	18	17	94.44	350-2600	0.915	0.56	12.29
UBC825	ACACACACACACACACT	18	18	100	300-2500	0.924	0.67	12.76
UBC 834	AGA GAG AGA GAG AGA GYT	16	16	100	400-2200	0.904	0.60	10.33
UBC 864	ATGATGATGATGATGATG	14	14	100	500-2500	0.895	0.70	10.52
UBC 900	ACTTCCCCACAGGTTAACACA	21	21	100	400-2500	0.931	0.46	9.71

Table 2. Contd.

UBC 824	TCTCTCTCTCTCTCTCG	18	18	100	450-2500	0.920	0.33	5.86
UBC 886	VDVCTCTCTCTCTCTCT	16	16	88.89	200-2100	0.889	0.60	10.81
UBC 889	DBDACACACACACACAC	19	19	100	150-1600	0.933	0.72	13.67
UBC 890	VHVGTGTGTGTGTGTGT	18	18	100	200-2200	0.923	0.76	14.52
UBC 895	AGAGTTGGTAGCTCTTGATC	17	17	100	200-3000	0.921	0.80	13.62
UBC 873	GACAGACAGACAGACA	21	21	100	400-2200	0.924	0.60	12.62
UBC 880	GGAGAGGAGAGGAGA	20	20	100	250-1800	0.902	0.44	9.76
Total		479	473	98.18	Average	0.91	0.54	10.47

R: GA (Purine); Y: TC (Pyrimidine); V: GCA (All but T); H: ACT (All but G); D: GAT (All but C); B: GTC (All but A).

Table 3. Genetic variability within population of *S. chirayita* as shown by POPGENE using ISSR-PCR primer data.

Population of *S. chirayita*	Sample size	NPB	PPB (%)	Na	Ne	H	I
Phulchowki, Lalitpur	9	173	72.90	1.7362	1.474	0.2672	0.3947
Kaski	4	109	51.42	1.507	1.2961	0.1749	0.2643
Sankhuwasabha	6	219	78.21	1.7821	1.4597	0.2706	0.4073
Terhathum	8	181	77.35	1.7702	1.4419	0.2589	0.3900
Nagarjun, Kathmandu	7	143	63.9	1.6356	1.3726	0.2192	0.3296
Illam	8	165	77.47	1.771	1.489	0.2801	0.4159
Average	-	-	70.20	-	-	0.2451	0.3669
Species level	42	473	98.18	1.9865	1.4514	0.276633	0.423056

generated altogether, 479 unambiguous and reproducible bands, of which 473 (98.18%) were polymorphic, the sizes ranged from 150 to 3000 bp. The numbers of bands varied from 8 to 24, with an average of 18 bands per primer. Polymorphism information content (PIC) score of primers under study ranged from 0.805 (UBC830) to 0.938 (UBC836) with an average of 0.91. The average band informativeness (I_B) of the 27 primers was 0.54 and it ranged from 0.31 (UBC 812) to 0.80 (UBC 895). Whereas, the resolving power (R_P) ranged from 3.43 for primer UBC 830 to 14.95 for primer UBC 841 with an average of 10.47.

Genetic diversity within populations

The percentage of polymorphic loci (PPB) ranged from 51.42 to 78.21%, with an average of 70.20% in individual populations (Table 3). Nei's gene diversities (H) varied from 0.174 to 0.270, with an average of 0.245, and Shannon's indices (I) ranged from 0.264 to 0.407, with an average of 0.366. In this study, the high genetic diversity was found in Sankhuwasabha populations (H and I values of 0.270 and 0.407, respectively), while low genetic diversity was found in Kaski populations (H and I values of 0.174 and 0.264, respectively). The genetic diversity of populations from high to low ranked as follows: Sankhuwasabha > Ilam > Terhathum > Phulchowki > Nagarjun > Kaski. It was also evidenced from the number (Na) and effective number of alleles (Ne) (Table 3). At species level, the H and I values equaled 0.276 and 0.423, respectively, and the Na and Ne values equaled 1.986 and 1.451, respectively.

Genetic construction of populations

The considerable level of genetic differentiation was observed among various populations of *S. chirayita* studied. The total gene diversity (H_T) and gene diversity within populations (H_S) were 0.278 and 0.120, respectively. The coefficient of genetic differentiation (G_{st}) amongst inter-populations of *S. chirayita* 0.548 indicated 54.8% variation in inter-populations and 45.2% variation within the populations (Table 4). AMOVA showed 48.0% genetic variation in inter-populations (Table 5); which strongly supports the result shown in genetic differentiation of *S. chirayita* as it was affected more in inter-population units. The gene flow was estimated to be 0.482. The correlation between genetic distance and geographical distance (r) value was found to be 0.418 ($p<0.001$), which indicated no significant correlation between the two matrices, based on genetic and geographical distances. F_{ST} value was found to be 0.6529.

Table 4. Genetic differentiation and diversity within and between the populations of *S. chirayita.*

Primers code	H_T	H_S	G_{ST}	N_m
UBC811	0.2719	0.1065	0.6084	0.3218
UBC842	0.2807	0.1432	0.4900	0.5204
UBC 810	0.2664	0.1113	0.5381	0.4292
UBC 820	0.2399	0.1154	0.5191	0.4632
UBC 830	0.2633	0.0732	0.7220	0.1926
UBC812	0.1613	0.1153	0.2850	1.2545
UBC 817	0.3370	0.0865	0.7433	0.1727
UBC 809	0.3033	0.0569	0.8123	0.1155
UBC 807	0.3331	0.1274	0.6176	0.3096
UBC836	0.2821	0.1432	0.4923	0.5156
UBC 848	0.254	0.0998	0.6072	0.3235
UBC 850	0.2667	0.1718	0.3557	0.9055
UBC 818	0.1671	0.0988	0.4088	0.7230
UBC 840	0.2986	0.1484	0.5032	0.4937
UBC 841	0.2874	0.1453	0.4942	0.5117
UBC 857	0.3117	0.1091	0.6500	0.2692
UBC825	0.3483	0.0674	0.8066	0.1199
UBC 834	0.2935	0.1174	0.6001	0.3331
UBC 864	0.3174	0.1941	0.3884	0.7872
UBC 900	0.2470	0.1444	0.4154	0.7036
UBC 824	0.1746	0.1253	0.2825	1.2701
UBC 886	0.2445	0.1022	0.5820	0.3590
UBC 889	0.3877	0.1340	0.6542	0.2643
UBC 890	0.3584	0.0986	0.7249	0.1897
UBC 895	0.3319	0.1517	0.5431	0.4207
UBC 873	0.2742	0.1428	0.4790	0.5439
UBC 880	0.2176	0.1116	0.4873	0.5260
Mean	0.2785	0.1200	0.5485	0.4829
Standard deviation	0.0561	0.0311	0.1432	0.2953

Table 5. Analysis of molecular variance (AMOVA) for 42 individuals in six populations from three regions [degree of freedom (d.f.), sum of squares (SS), mean square (MS), estimated variance, percent (%) and its associated significance (n=999 permutations)].

Source of variation	d.f.	SS	MS	Estimated variance	Total variance (%)	P-value
Among populations	5	1904.740	380.948	47.658	48	<0.01
Within populations	36	1835.117	50.975	50.975	52	<0.01
Total	41	3739.857	-	98.633	100	-

Cluster analysis based on the ISSR genotyping profiles

The results from Mantel test (Matrix comparison) using NTSYS-PC (Version 2.21i) showed that the correlation between Jaccard and Dice similarity matrices was the highest and significant (0.99252) (Table 6 and Plate 1). Clustering based on unweighted pair group method of arithmetic averages (UPGMA) for Jaccard coefficient was observed to give a high cophenetic correlation value of 0.96875 and comparatively lowest cophenetic correlation value of 0.94904 was observed for UPGMA clustering using simple matching coefficient. Because of their highest correlation value and comparison of standard chart of goodness of fit, Jaccard's coefficient of similarity with UPGMA clustering method was the best for studying relationship among *S. chirayita* accessions. In the study, Jaccard similarity with UPGMA yielded the highest correlation coefficient value but the difference between Jaccard, Dice and simple matching coefficients was not

Table 6. Correlation coefficients from Mantel test (2 way) of original matrices.

Correlation parameter	Simple matching	Jaccard	Dice
Simple Matching	****	-	-
Jaccard	0.92823	****	-
Dice	0.90743	0.99252	****

Table 7. Correlation coefficient value (r) obtained from cophenetic values of similarity matrices (simple matching, Dice and Jaccard's coefficient) and clusters computed by UPGMA module using MXCOMP (matrix comparisons) option of NTSYS.

Clustering module of similarity	Simple matching	Dice	Jaccard
UPGMA	0.94904	0.95197	0.96875

Table 8. Consensus fork index (CI_C) among the UPGMA based phenograms produced by similarity coefficients among *S. chirayita* accessions by ISSR marker.

Correlation parameter	Jaccard	Dice	Simple matching
Jaccard	****	0.9805	0.7500
Dice	-	**_**	0.7500
Simple Matching	-	-	****

so far (0.96875, 0.95197 and 0.94904 respectively). From this test, the three coefficients were in the decreasing order as J > D > SM (Table 7). Consensus indices (CI) were calculated for each combination of coefficient and UPGMA clustering for the evaluation of trees constructed from UPGMA clustering by genetic similarity coefficients. Highest Consensus fork index (CI_c = 0.9805) was found for Jaccard and Dice coefficients. The CI_c values for J and SM and D and SM were lower (CI_c = 0.7500) (Table 8).

Based on the Jaccard's similarity matrix (01-0.86) clarified the genetic relationship of *S. chirayita* accessions from geographically diverse population. Individuals (42) were found separated into two major clusters (Clusters I and II) and six sub clusters A, B, C, D, E and F at the similarity level of 0.24 (Figure 2). The sub clusters A and B were separated at the similarity coefficient level of 0.27. Likewise, the clusters C and D were separated from A and B at the similarity coefficient of 0.25. The sub-cluster A contained accessions from Phulchowki and Kaski. All accessions (except F9) from Phulchowki were clustered at the similarity coefficient level of 0.65, while F9 accession was clustered at the similarity coefficient level of 0.425. Likewise, all the individuals from Kaski were clustered as the similarity coefficient level of 0.68. The sub cluster B contained accessions from Nagarjun and were clustered at the coefficient level of 0.62. The sub cluster C contained accessions from Sankhuwasabha (S1 and S2), whereas sub cluster D contained accessions from both Sankhuwasabha (S4, S5, S6, S7 and S8) and Terhathum. The accessions from Sankhuwasabha, S1 and S2 were separated from rest of the individuals at the similarity coefficient level of 0.35, while the rest of individuals were clustered at the level of 0.675. The accessions T7 and T6 were separated from the rest of the individuals of Terhathum at similarity coefficient level of 0.52 and 0.50, respectively. The sub clusters E and F contained accessions from Ilam. The individuals of Ilam were clustered at the similarity coefficient level of 0.49 except the accession I4 at 0.345. The PCoA analysis was carried out based on the Euclidean matrix. In the plot, first (percentage of variance = 18.33%) and second (percentage of variance = 16.90%) axis with a cumulative variance of 35.23% was seen. The PCoA plot supports the result of dendrogram by clustering the individuals according to their geographical locations (Figure 3). It showed the congruence with 3D-plot of the distribution of all *S. chirayita* accessions (Figure 4).

DISCUSSION

The genetic polymorphism observed in the present study for *S. chirayita* was very high (that is, 98.18%) as compared to the result from 13 Indian *S. chirayita* genotypes from temperate Himalaya (42.5%) using ISSR marker (Joshi and Dhawan, 2007). When over 50% of the total genetic variation existed within populations, six populations of species should retain 95% of their genetic

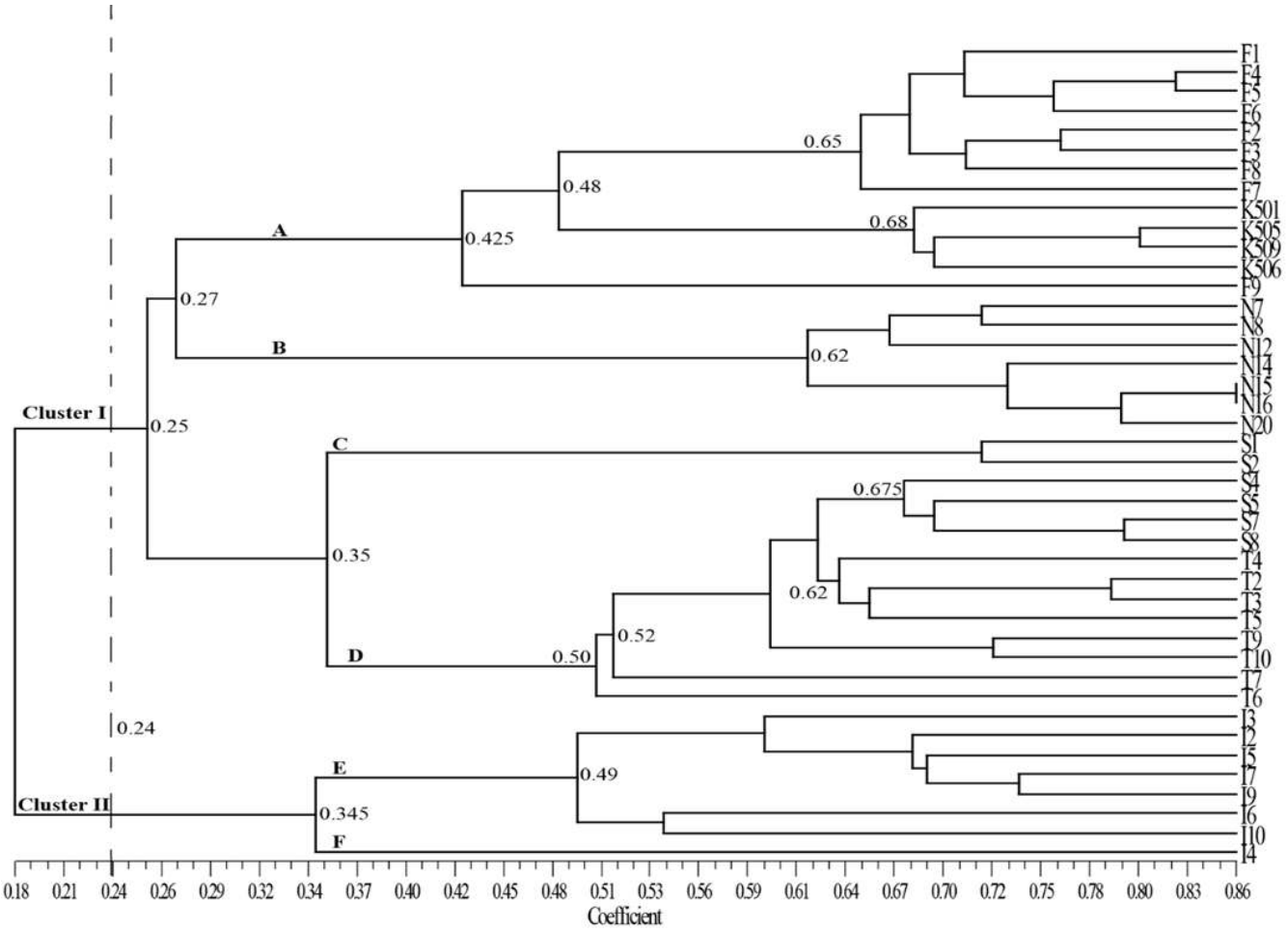

Figure 2. Dendrogram generated for 27 polymorphic ISSR-PCR primers data of 42 *Swertia chirayita* accessions using Jaccard's similarity coefficient by UPGMA method of Cluster analysis. The clusters are labeled as A, B, C, D.

diversity (Hamrick and Murawski, 1991). The investigation showed substantial genetic differentiation among *S. chirayita* populations. However, RAPD based analysis of 34 accessions of *S. chirayita* (used in this present research) revealed 92.28% polymorphism (Shrestha et al., 2013). *S. tetraptera*, an endemic species of Qinghai-Tibetan Plateau revealed 98.9% polymorphism using ISSR fingerprinting (Yang et al., 2011) and *S. przewalskii* from the same region showed 56% polymorphism using ISSR marker (Zhang et al., 2007). Genetic polymorphism shown with ISSR analysis for *Neopicrorhiza scrophulariiflora* also showed high level of polymorphism of 100% (Liu et al., 2011), likewise, 100% for *Dendrobium* studies (Wang et al., 2009). The high level of both polymorphism and reproducibility is observed using ISSR-PCR technique because of the use of longer primers which increases stringency in annealing temperatures than in RAPD-PCR technique (Kojima et al., 1998).

The present study revealed Jaccard's similarity coefficient values ranging from 0.1 to 0.86. This values suggest the presence of high genetic diversity within *S. chirayita* species of six populations. Similar result was observed among the *S. chirayita* species collected from temperate Himalaya of India as Jaccard's coefficient was observed in the range of 0.68 to 0.97 (Joshi and Dhawan, 2007). Although, there was a different cluster pattern in PCoA, the Mantel test revealed no significant correlation between genetic distance and geographical distance ($r = 0.418$; $p < 0.001$). This might be due to clustering of accessions from Phulchowki and Kaski together, which are approximately 200 km apart. The accessions from Ilam were grouped together in a separate cluster.

For the formulation of conservation strategies, understanding of the diversity of threatened species is quintessential (Kareem et al., 2012). The species should possess enough genetic variability as it plays important role in adaption in the changing environment (Schaal et al., 1991). The polymorphism study can be shown in terms of the Nei's genetic diversity (H), Shannon's information index (I), total heterozygosity (H_T), average heterozygosity (H_S), coefficient of population differentiation

Axis	1	2	3
Percentage	18.33	16.90	12.97
Cumulative Percentage	18.33	35.23	48.20

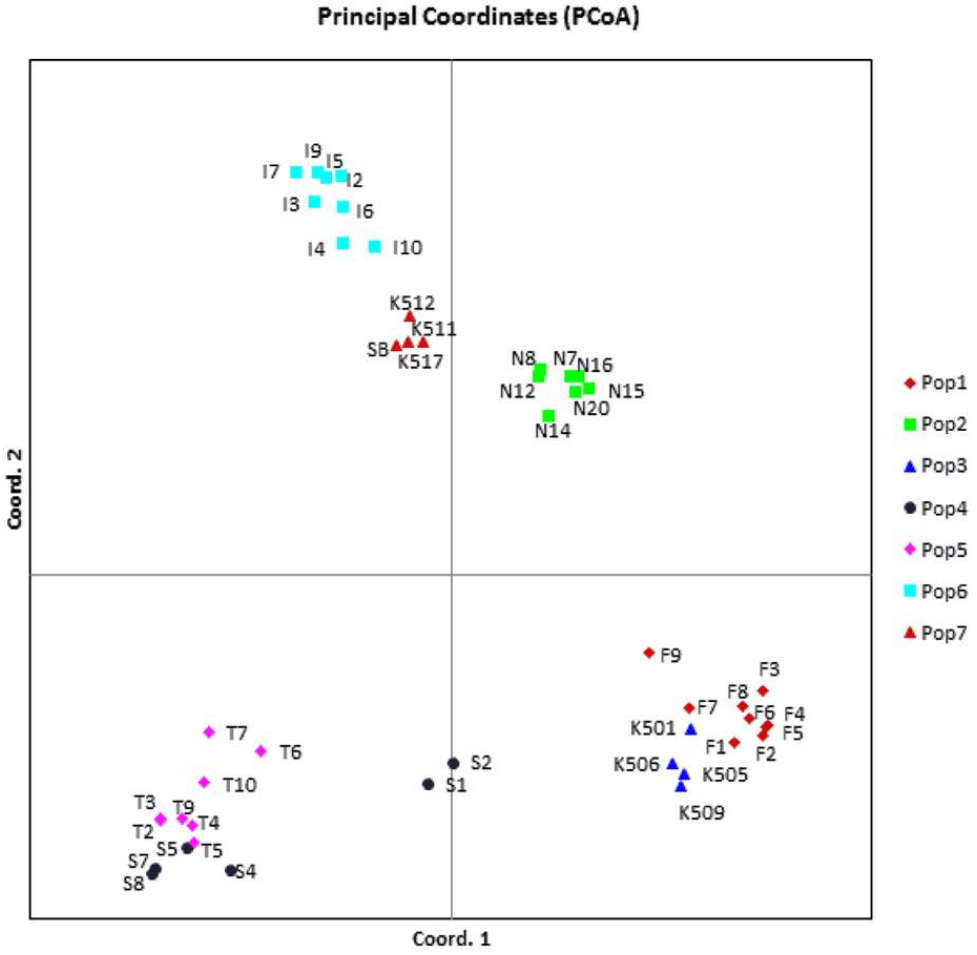

Figure 3. Two-dimensional scatter plot of PCoA axis generated for 42 *Swertia chirayita* accessions by 27 ISSR-PCR primers through Principal Co-ordinate Analysis.

(G_{ST}) and gene flow (N_m) (Zhao et al., 2006). The value of gene differentiation (G_{ST}) ranging < 0.05, 0.05 to 0.15 and > 0.15 is grouped as low, medium and high population differentiation, respectively (Nei, 1978). Also, the value of gene flow (Nm) < 1 which denotes less than one migrant per generation into a population is the threshold value at which the differentiation occurs in population in a significant amount (Slatkin and Barton, 1989). When the condition arises where N_m is found to be less than one, it is suggested that the diversity maintained in the population is prone to genetic drift (Wright, 1949). High G_{ST} value (0.5485) and the low N_m value (0.4829) were observed in the present study and showed rapid genetic differentiation among the six populations of *S. chirayita*. The reason behind the high level of population differentiation can be the geographic separation of the populations (Hogbin and Peakall, 1999). The genetic variation in *S. chirayita* could be due to genetic drift within the population. Also, effect of the gene flow among inter-populations of *S. chirayita* is not significant. The genetic heterogeneity showing index, the Shannon's index was observed to be the highest (0.416) for accession from Sankhuwasabha along with high Nei's gene diversity index (0.280), whereas the lowest Shannon's index (0.0.264) was found for Kaski accession with low Nei's gene diversity index (0.175) and PPB (51.42%). Study of species level diversity showed high level of genetic differentiation with 98.18 of PPB and high Shannon's index of 0.423, which shows the high polymorphism in the *S. chirayita* populations.

The data on the genetic structure of *S. chirayita* obtained in the present study suggest that the differentiation coefficients (G_{ST}=0.5485 and F_{ST}=0.6529) are higher than the average coefficients of outcrossing

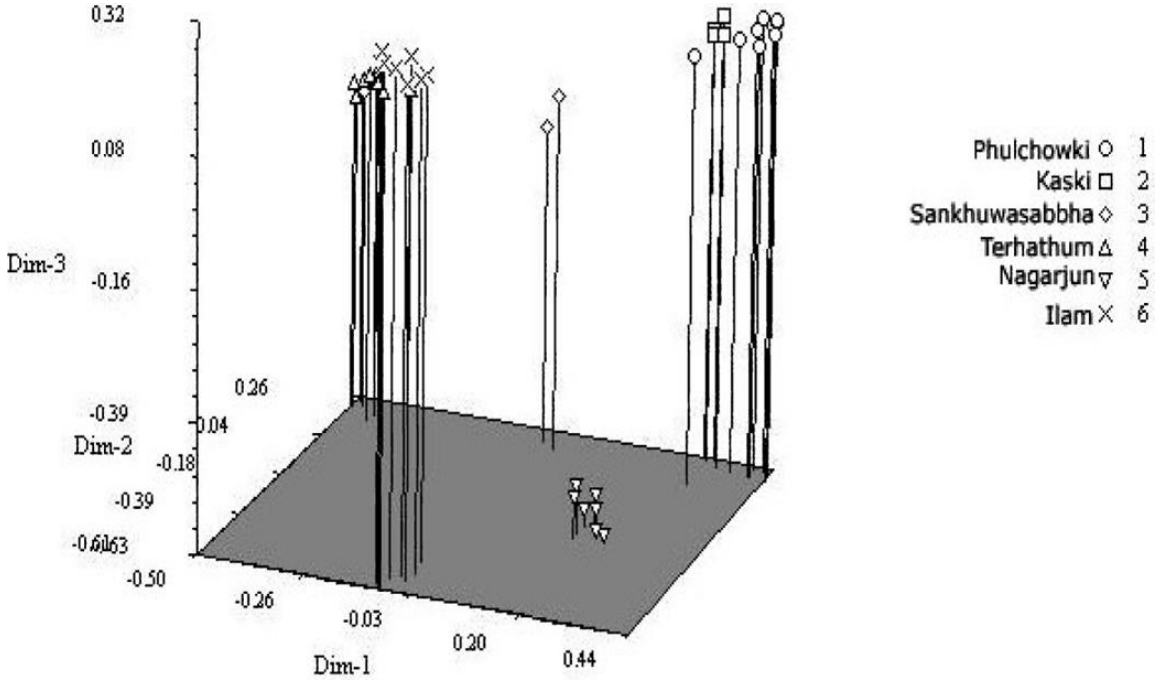

Figure 4. Three-dimensional plot constructed using NTSYS-PC ver2.2i to assess the dispersion of *S. chirayita* accessions.

species (G_{ST}=0.22 and F_{ST}=0.27) and similar to the average coefficients of inbreeding species (G_{ST}=0.59 and F_{ST}=0.65) (Nybom, 2004). High genetic differentiation in this species may suggest that the individual populations have been reproductively isolated and there is little current gene flow between them. The result is in agreement with Soumendra et al. (2009) in which *S. chirayita* has demonstrated the self-pollination in natural pollination of formation of seeds. However, it contrasts with the aspect of high genetic diversity observed among the *S. chirayita* individuals of different populations that indicate the existence of significant cross-pollination among the individuals in the population. The natural environment of the plants from Gentian family shows the probability of out crossing by 16 to 20% (Dudash, 1990). The pollinators such as bees and insects are responsible for cross pollination as they collect nectar from nectar glands (Khoshoo and Tandon, 1963). The morphological reason behind the self-pollination can be the structure pattern of androecium and gynoecium (Kulkarni et al., 2005). In *S. chirayita*, the distance between the anther sac and stigma is less which creates the condition for self-pollination (Proctor et al., 1996). Also, the various studies revealed that *S. chirayita* is mostly cross pollinated with potential of self-pollination (Shah et al., 2011; Raina et al., 2013).

Conclusion

S. chirayita is considered a highly valued medicinal plant of Nepal. In the present investigation, microsatellite based ISSR technique was employed for the assessment of existing genetic diversity among six *S. chirayita* populations from eastern, central and western regions of Nepal. The conservation of *S. chirayita* populations *in situ* to preserve its genetic diversity is suggested. The findings provide insights into important genetic information for formulating and effecting conservation strategies and cultivation of *S. chirayita*. Easily identifiable and confusion with the other *Swertia* spp. twinned with capacity of possessing chemical compounds from the early stage are the main reasons for premature harvesting of *S. chirayita*. This has resulted into the major problem as seeds could not be dispersed in the natural environment. *In vitro* tissue culture technique to be followed in order to reduce harvesting pressure on wild populations of *S. chirayita* is suggested. Additionally, to implement effective conservation strategies of *S. chirayita*, it is crucial to understand species pollination, breeding system associated with the genetic structure.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

This project was supported by Ministry of Science and Technology, Government of Nepal. The authors

acknowledge Nepal Academy of Science and Technology (NAST) for supporting this project, the members of Molecular Biotechnology Laboratory, NAST, Jagat Krishna Chhipi Shrestha, Smita Shrestha, Dr. Ranjan Koirala, Dr. Ram Chandra Poudel, Dinesh Neupane, Sunita Khanal, Nabin Narayan Munankarmi, Jyoti Bhuju, Bibechana Adhikari, Rajesh Lamichhane, Gaurav Gyanwali, Nisha Rana and Dr. Jyoti Amatya for their kind cooperation and help.

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